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Patents ADP number *(if you know it)* 3992564001

If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. Title of the invention ANTIBODY VARIANTS

5. Name of your agent *(if you have one)* STEVENS, HEWLETT & PERKINS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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# ANTIBODY VARIANTS

This invention relates to modified antibodies for inducing immunological tolerance in human beings or animals.

Antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, each light chain being linked to a respective heavy chain by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the light chain variable domain being aligned with the variable domain of the heavy chain and the light chain constant domain being aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. The variable domains of the light and heavy chains have the same general structure; each domain comprises four framework regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The CDRs are held in close proximity by the framework regions. CDRs from adjacent light and heavy chain variable domains together contribute to the formation of the antigen binding site.

Background of the Invention

Antibodies directed to specifically chosen antigens have been used in the treatment of various conditions. For example, Campath-1 monoclonal antibodies (mAb) have been used successfully to induce remissions in lymphoma and leukemia patients and for the treatment of rheumatoid arthritis and vasculitis. The target antigen, CD52, is a GPI-

anchored glycoprotein of lymphocytes and monocytes (and parts of the male reproductive system). It has an exceptionally short peptide sequence of 12 amino acids and a single, complex, N-linked oligosaccaride at Asn3 (Hale et al, 1990; Xia et al, 1991). CD52 is a good target for antibody-mediated killing and is therefore an effective cell surface molecule for various therapeutic regimens in which reduction in lymphocytes is an objective (e.g. removal of cells from donor bone marrow to prevent graft-versus-host disease, treatment of leukemia and lymphoma, and immuno-suppression).

Several rat anti-human CD52 Campath-1 mAb were generated by fusion of the Y3 rat myeloma line with spleen cells from a rat immunized with human T lymphocytes (Hale et al, 1983). Although the clinical effectiveness of rat Campath-1 mAb have been demonstrated regularly, many patients mounted an anti-antibody (antiglobulin) response against the xenogenic protein that prevented retreatment with the therapeutic antibody. Antibody therapy is often limited by the antiglobulin response. The anti-idiotypic component (anti-Id; directed against the Ab-combining site) inhibits the binding of the Ab to its target while both the anti-Id and the anti-isotypic component (directed against the constant regions) act to accelerate antibody clearance. A major concern is the neutralizing effect of the antiglobulin response. As with antiglobulin responses in general, anti-id responses interfere with the clinical potency of a therapeutic Ab by forming Ab aggregates that are rapidly cleared from the circulation, reducing the chance for interaction with target antigen. Unfortunately, most antiglobulin sera contain anti-Id antibodies. This has been demonstrated for a number of therapeutic mAb and is especially noted after repeated treatments.

To reduce the immunogenicity of the rat IgG2b Campath-1 antibody, YTH34-5, the gene fragments encoding the VL and VH were humanized by "CDR grafting" of the rodent hypervariable regions onto

human framework regions (Jones et al, 1986; Reichmann et al, 1988). The humanized VL and VH were then genetically fused with human light chain and heavy chain constant regions, respectively. Therefore, the genetically constructed Campath-1 immunoglobulin light and heavy chains contained sequence that was human except in the 6 CDR regions that encode the antibody's specificity. In clinical trials, the humanized version (Campath-IH) was found to be much less immunogenic than the rat IgG2b Campath-1 antibody. Humanization reduces the immunogenicity of rodent mAb, although both the idiotype and the allotype of a humanized mAb might still be targets for humoral responses. Sensitization to idiotype has indeed been documented in some allotype-matched recipients of Campath-IH (Isaacs et al, 1992; Lockwood et al, 1993). These responses were revealed by the presence of anti-Id in the patients' sera. One patient generated high-titre anti-Id that crossreacted on the entire panel of CD52 mAb. Humanized Campath-1 antibodies are described in EP 0 328 404.

One strategy to further reduce the immunogenicity of Campath-IH might be to re-graft the 6 CDR loops onto well-characterized human germline framework regions. The majority of the humanized V regions so far have used rearranged V-genes as acceptor framework sequence. This was the case for Campath-IH as framework sequence from myeloma proteins were used to provide acceptor sequences for both VH and VL. Rearranged V-genes often contain somatic mutations, acquired during the process of affinity maturation. These will be unique to the individual from which the rearranged genes were derived and therefore may be seen as foreign in another individual. However, there is a possibility that regrafting may introduce new idiotypic epitopes, formed by the junctional regions encompassing CDR residues and new framework residues. Furthermore, humanization alone may not solve the problem of anti-Id responses because the human population is outbred and it is unlikely that all patients will be tolerant to a given humanized mAb. Even

in antibody constant regions, there are a number of different alleles which carry allotypic markers to which naturally occurring antiglobulin responses can be demonstrated. The problem is more complex for V-region segments, which show a higher degree of variation both in allotype and haplotype in comparison to constant regions.

Another approach is to induce tolerance to the potentially foreign peptides contained within the Campath-IH V-region. We know that the antiglobulin response is itself a B-cell response which is CD4+ T-cell dependent. Isaacs and Waldmann (1994) demonstrated that mice deprived of CD4+ T-cells were unable to respond to a foreign cell-binding mAb (rat anti-mouse CD8 mAb). CD4+ T-cell depletion was carried out by adult thymectomy combined with administration of a depleting CD4 mAb. In these mice, the response to subsequently administered mAb or SRBC was measured. CD4+ T-cell deficient mice failed to make either an antiglobulin response or an anti-SRBC response, demonstrating that the anti-Ig response, like the anti-SRBC response, is classically CD4+ T-cell dependent. In order to generate T-cell help and to get the appropriate T-cell response, the adminstered Ab must be processed as a protein antigen and presented, presumably in the context of an MHC class II molecule, by a suitable antigen presenting cell. Therefore, two main strategies can be adopted to decrease the immunogenicity of a humanized V-region. (1) We can "silence" the antibody molecule itself, adopting strategies to eliminate any potential T helper epitopes, or (2) we can present all the potential T helper epitopes in a manner that induces tolerance instead of reactivity to those epitopes.

**"Silencing" the antibody molecule:**

a) In theory, we might be able to silence the antibody itself so that the immune system will not recognize foreign determinants. This would be possible if we could scan the VL and VH amino acid sequences for motifs that could bind to MHC class II molecules. If we could thus

identify key residue(s) in a potential class II peptide that were not involved in antibody specificity or affinity, then it/they could be changed by site-directed mutagenesis to residue(s) that did not allow association with class II molecules. T helper peptides are not random, and any protein has only a limited number of peptides capable of binding to MHC class II molecules, and also to T-cell antigen receptors. However, this is not possible at present because class II-binding peptides are not yet characterized to a sufficient degree to be identified by scanning protein sequence. This is in part due to the heterogeneous nature of class II peptides. Naturally processed peptides isolated from MHC class II molecules are generally larger in size, variable in length and have both ragged ends at C- and N-termini in comparison to processed peptides isolated from MHC class I molecules. Whereas class I-derived peptides are mostly of uniform length of 8-9 amino acid residues, MHC class II-associated peptides range from 12-24 amino acids (Rudensky et al, 1991; Hunt et al, 1992; Rudensky and Janeway, 1993). Class I-derived peptides have sequence motifs with specific anchor residues in certain positions allowing their side chains to fit in the binding pockets of the peptide-binding groove, and the peptide-binding groove is closed at both ends. In contrast, class II peptides are bound in an extended conformation that projects from both ends of an "open-ended" antigen-binding groove; a prominent non-polar pocket into which an anchoring peptide side chain fits near one end of the binding groove (Brown et al, 1993).

b) Other strategies that might be adopted to "silence" the antibody if we could predict class II peptide-binding motifs. For example, one could include insertion of a protease cleavage site within any potential class II epitope to increase the chance of peptide degredation before they could be presented in the context of class II. Alternatively, insertion of motifs into a V-region such as Gly-Ala repeats may inhibit the degradation of the V-region into peptides that could associate with class II molecules.

In one system, it was shown that EBNA1 Gly-Ala repeats generated a cis-acting inhibitory signal that interfered with antigen processing during MHC class I-restricted presentation such that CTL recognition was inhibited (Levitskaya et al, 1995). Although either of these approaches may hold some promise in the future, they again rely on prediction of potential MHC class II peptides from protein sequence of humanized VL and VH regions and are therefore limited by insufficient knowledge regarding consensus motifs for class II peptides.

**Inducing tolerance to T helper epitopes**

In lieu of sufficient knowledge regarding class II peptide motifs, we have turned our attention toward induction of tolerance to therapeutic antibodies. In 1986, Benjamin et al and Cobbold et al described an unexpected property of cell-binding mAb: whereas it was possible to induce tolerance to the Fc region (anti-isotype tolerance), the idiotype remained antigenic under equivalent conditions. Moreover, it was relatively easy to induce tolerance to non-cell binding mAb but cell-binding mAb were found to be very immunogenic.

Isaacs and Waldmann (1994) in a preliminary study used a non-cell-binding variant of a "therapeutic" Ab to induce tolerance to the wild-type form (in this case, "therapeutic" refers to a cell-binding anti-CD8 mAb in a mouse model). The non-cell-binding Ab was made by pairing the relevant L- and H-chains with an irrelevant H- or L-chain, respectively. The relevant H-chain paired with an irrelevant L-chain was obtained by limiting dilution cloning of the original hybridoma that was expressing a myeloma light chain (from the Y3 fusion partner), as well as the specific anti-CD8 H- and L-chains. A variant of the hybridoma that expressed the myeloma L-chain and the specific anti-CD8 H-chain but no anti-CD8 L-chain was obtained. A clone expressing the relevant L-chain only was also obtained in this manner. That clone was then fused to a hybridoma expressing an irrelevant specificity (anti-human CD3) and a variant was selected that

expressed the relevant anti-CD8 L-chain with the irrelevant anti-CD3 H-chain. Because proteins are processed into peptides prior to presentation to T-cells, helper peptides from antigen-specific H- and L-chains would be "seen" by T-cells, regardless of their partner chain. However, in this case, there was no advantage in tolerance induction using non-cell-binding variants of a therapeutic mAb in vivo compared to an isotype-matched control, suggesting that in the strain of mice used, most (or all) of the helper epitopes were located within the constant region.

In practice, using these "mixed molecules" of antigen-specific and irrelevant immunoglobulin chains for human therapy would not be feasible because the irrelevant H- and L-chains would presumably carry some helper epitopes themselves, thus complicating the ability to achieve tolerance to the relevant H- and L-chains. Nor would one expect to tolerize those B-cells which "see" idiotypic determinants formed by the combination of the relevant H- and L-chains of the antibody.

Campath-1 is a cell-binding mAb, and an effective tolerogen for use with it, such as a non-cell-binding form of the therapeutic mAb would therefore be advantageous. The same goes for other therapeutic antibodies which have cell-binding properties.

**The Invention**

The invention therefore provides an antibody which is a modified version of a therapeutic antibody with affinity for a cell-surface antigen, said antibody having reduced affinity for the antigen compared with the therapeutic antibody as a result of a modification, wherein the constant region of the antibody substantially corresponds to the constant region of the therapeutic antibody and the modification comprises an alteration in one of the complementarity determining regions (CDRs).

Preferably, the affinity of the antibody according to the invention for the antigen is reduced to 50% or less of the affinity of the therapeutic antibody for the antigen. More preferably, the affinity is

reduced to 10% or less, or to 1% or less of the affinity of the therapeutic antibody. The affinity needs to be sufficiently reduced to allow the antibody according to the invention to act as a tolerogen with respect to the therapeutic antibody. The term "non-cell-binding variant" is used herein to refer to antibodies according to the invention, although antibodies according to the invention may still have some binding affinity for the cell surface antigen.

The antibody according to the invention is preferably as close as possible to the therapeutic antibody on which it is based. Administration of such a "minimal mutant" prior to injection of the cell-binding therapeutic mAb would be expected to tolerise to all T- and most B-cell epitopes in the therapeutic mAb. Classic experiments indicate that tolerance is maintained more effectively by T- cells than by B-cells. But since most B-cell responses including the anti-Id response require T-cell help, even if a B-cell is responsive to a given antigen, antibody production will be determined by the state of responsiveness of the T-cells (Chiller et al, 1971). Thus, it will be preferable to use a non-cell-binding variant which contains the minimum differences needed to reduce its affinity for the cell-surface antigen sufficiently to enable it to be used as a tolerogen. By using techniques such as X-ray crystallography, computer modeling and site-directed mutagenesis, it will be possible to design suitable non-cell-binding variants for any cell-binding therapeutic antibody.

The antibody according to the invention is preferably in biologically pure form, desirably being at least 95% (by weight) free of other biological materials.

In one particular embodiment of the invention, the non-cell-binding antibody is in monovalent form. A schematic representation of a monovalent antibody according to the invention is shown in figure 1. The monovalent antibody has a single antigen-binding site instead of the usual two, which further reduces affinity of the antibody for its antigen.

As used herein, the term "cell-surface antigen" means an antigen which is found on cell surfaces, but not necessarily exclusively on cell surfaces.

The term "therapeutic antibody" is used herein to refer to an antibody which may be administered to humans or animals to have a desired effect in particular in the treatment of disease. Such therapeutic antibodies will generally be monoclonal antibodies and will generally have been genetically engineered.

In another aspect the invention comprises a composition for administration to a patient comprising an antibody as described herein, together with a physiologically acceptable diluent or carrier.

In a further aspect, the invention provides a cell line which expresses an antibody as herein described.

Additional aspects of the invention include the use of an antibody as described herein in the manufacture of medicament for use in a treatment which requires induction of tolerance to a therapeutic antibody, as well as such a method of treatment.

In yet another aspect, the invention provides a monovalent version of a therapeutic antibody for use in the induction of tolerance to the therapeutic antibody. The reduction in binding affinity of a monovalent antibody compared to its bivalent counterpart may be sufficient to enable tolerance induction. Preferably, the monovalent antibody is either incapable of binding Fc receptors, or incapable of binding complement component C1q, or both. Either or both of these properties can be introduced by suitable mutations and will contribute to the reduction in antigen binding affinity of the monovalent antibody.

The invention will now be further described in the examples which follow.

## EXAMPLE

### A. Using rational design to create a "minimal mutant"

1. Identification of key residues for antigen binding:

The first requirement in producing a non-cell-binding variant of the therapeutic mAb is to determine which amino acid residues are involved in binding to target antigen. Relative to the number of residues that comprise the VL and VH domains, those that are directly involved in interactions with antigen are small in number (Novotny et al, 1983). And although the Ab-combining site is made up of 6 hypervariable loops, 1 or 2 of those loops may dominate in that interaction. If a key residue or residues can be identified, it/they can be changed by site-directed mutagenesis to a residue that will abolish antigen-binding. Because these residues will most likely be found within the hypervariable loop structures and not in the framework sequence supporting those loops, small changes may not significantly disrupt the overall structure of the Ab.

We have devised a method to determine which of the CDR loops of the humanized Campath-1 mAb are the most important ones for binding to CD52. Mutant V-regions were genetically constructed in which each of the 6 hypervariable regions was individually swapped for the corresponding CDR from the V-region that had provided the human VL or VH acceptor sequence during humanization (REI and NEW, respectively). The engineered V-regions were expressed as Fab fragments in E coli using the pHEN vector (Hoogenboom et al, 1991). In this system, the pelB leader sequence was used to direct protein expression to the periplasm, where association of L-chain and truncated H-chain occurs (Hoogenboom et al, 1991). When these Fab fragments were assayed for binding to immobilized CD52, it was found that swapping the (VH) CDR2 of NEW into the humanized Campath-1 Fab completely destroyed binding to CD52. Replacing (VH) CDR3 reduced binding to CD52 8-fold while replacing (VH) CDR1 and (VL) CDR3 reduced binding 3-fold. No change in binding was

detected when (VL) CDR1 or (VL) CDR2 were replaced. From these results, it appeared that (VH) CDR2 contained a key residue(s) necessary for antigen-binding.

The fact that 3 individual Campath-I mAb have different residues at H53 suggests that we may be able to make additional mutations at this site without destroying the overall structure of the V-region.

2. Mutation of key residue(s):

The wild-type humanized Campath-I H-chain was used as PCR template for site-directed mutagenesis. The two changes (mutation 1 is LysH54 to Asp and mutation 2 is AspH53 to Lys) were encoded on oligonucleotide primers and were inserted into an intermediate vector (PUCI9) for sequence verification. A unique HindIII site upstream of the VH leader sequence and a unique BstXI site in CH1 allowed the mutant V-region fragments (and partial CH1 sequence) to be isolated as HindIII-BstXI fragments for transfer back into the original VH construct by replacing the wild-type Hindm-BstXI sequence. These 2 mutagenized H-chain constructs are now ready for transfer into the Campath-I H-chain expression vector. Both will be expressed as co-transfections with the wild-type humanized Campath-I L-chain. Assuming the proteins are expressed, folded and secreted as for wild-type humanized Campath-I mAb, the minimal mutants will be tested for altered binding to CD52.

**B. In vivo models of tolerance induction:**

To test whether we can use either of the minimal mutants of Campath-I to tolerize to the wild-type Campath-I mAb in vivo, we can use transgenic mice. These mice express human CD52 behind a murine CD2 promoter to mimic the expression of CD52 on T cells. These mice should produce an anti-globulin response including an anti-Id component to humanized wild-type Campath-I mAb as the constant regions and framework regions will be of human origin whereas the CDR loops are of

rat origin. The effectiveness of the minimal mutants in tolerizing to subsequent challenge of wild-type Campath-I mAb will then be assessed.

**C. How the strategy might be adopted for human therapy:**

500 mg of the non-cell-binding form of the therapeutic antibody would be freshly deaggregated (for example, by passage through a fine filter) and administered to a patient awaiting treatment with the humanized Campath-1 antibody. Seven days later, during which time the T-cells and B-cells would become tolerized, the wild-type form of the therapeutic antibody would be given.

1:). Additional considerations:

1. It should be easier to tolerize to a minimal mutant than to HGG or to mixed chain Ab molecules.

In the tolerance models of Benjamin et al (1986), tolerance to polyclonal HGG was induced in mice following depletion of CD4+ T-cells, but also using deaggregated material. It was found that tolerance to these soluble proteins could be achieved relatively easily. Also, in the work of Isaacs and Waldmann (1994), CD4 Ab were given during tolerance induction to the non-cell-binding mixed chain Ab molecules (irrelevant and antigen specific H- and L-chains), or non-cell-binding forms were used as tolerogens in their own right following their deaggregation.

In our modified approach to inducing tolerance using a minimal mutant, the foreigness of the protein will be less than that of polyclonal HGG or of the mixed chain Ab molecules in mice. Since the therapeutic mAb is humanized, only the CDR loops are comprised of rodent sequence. It therefore may be possible to tolerize with a deaggregated minimal mutant in the absence of CD4 mAb. However, even if CD4 administration was required, a humanized therapeutic CD4 is available (CAMPATH-9; Gorman et al, 1991). The studies in transgenic animals should address these details.

2. Creation of a monovalent form of the minimal mutant.

Thus far we have considered tolerance induction using a minimal mutant that is essentially like the wild-type therapeutic except for minimal residue change(s) that will disrupt antigen binding. In practice, it may be difficult to completely abolish binding of the Ab to CD52. We have therefore considered making a monovalent form that is also significantly smaller than the minimal mutant. It is a single-chain Fv (formed by the VL, a (Gly4Ser)3 amino acid linker (Bird et al, 1988; Huston et al, 1988), and the mutated VH) genetically fused with sequence encoding the hinge-CH2-CH3 of human IgG. This construct is expressed in association with a truncated heavy chain (hinge-CH2-CH3 only; Routledge et al, 1991) such that a protein is expressed that is composed essentially of a single Ab-combining site and a functional human Ig Fc region. The (Gly4Ser)3 linker is unlikely to be immunogenic in vivo as it is composed only of a fifteen amino acid sequence of a Gly4Ser repeat. SFv have been expressed in mammalian cells from a number of different antibodies and have been shown to fold into the correct conformation for antigen-binding by functional activity (Gilliland et al, 1996). The Fc portion should ensure serum half-life comparable to the minimal mutant and to the wild-type therapeutic Ab, whilst monovalency will ensure that binding to CD52 is greatly reduced due to the decrease in avidity. We have already shown from the CDR-swapping experiments (section A1) that the Campath-1 Ab binds poorly to CD52 in a monovalent form. In addition to reducing the avidity of the molecule, the smaller size may be a bonus: in classical tolerance experiments, it was found that the smaller the molecule, the better it was at inducing tolerance (Parish and Ada, 1969; Anderson, 1969; Miranda et al, 1973). By combining monovalency with the non-cell-binding mutant, we could increase our chances of obtaining an effective tolerogen.

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## CLAIMS

1. An antibody which is a modified version of a therapeutic antibody with affinity for a cell-surface antigen, said antibody having reduced affinity for the antigen compared with the therapeutic antibody as a result of a modification, wherein the constant region of the antibody substantially corresponds to the constant region of the therapeutic antibody and the modification comprises an alteration in one of the complementarity determining regions (CDRs).

2. An antibody as claimed in claim 1, wherein the alteration is achieved by genetic manipulation of a nucleic acid coding for the CDR.

3. An antibody as claimed in claim 1 or claim 2, wherein the affinity of the antibody for the antigen is reduced to 50% or less of the affinity of the therapeutic antibody.

4. An antibody as claimed in any one of claims 1 to 3, wherein the CDRs are foreign with respect to the constant region of the antibody.

5. An antibody as claimed in claim 4, wherein the CDRs are foreign with respect to the heavy and light chain variable domain framework regions.

6. An antibody as claimed in claim 5, which is of human origin other than the CDRs.

7 An antibody as claimed in any one of claims 1 to 6, wherein the alteration is a change in a single amino acid.

8. An antibody as claimed in any one of claims 1 to 7, wherein the therapeutic antibody has affinity for CD52.

9. An antibody as claimed in claim 8, wherein the alteration is in (VH) CDR2.

10. An antibody as claimed in claim 9, wherein the alteration is a substitution of amino acid H54 and/or H/53.

11. An antibody as claimed in any one of claims 1 to 10, which is

monovalent.

12. An antibody as claimed in any one of claims 1 to 11, for inducing tolerance to the therapeutic antibody in a patient.

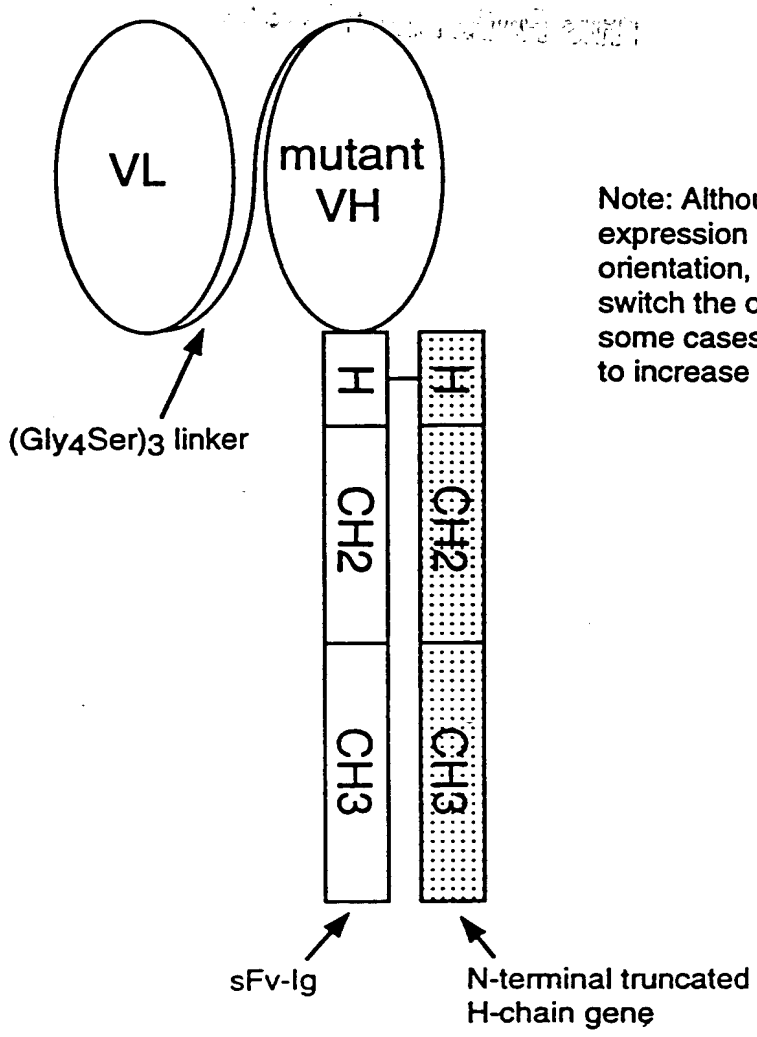
13. A cell line which expresses an antibody as claimed in any one of claims 1 to 12.

14. A composition for administration to a patient comprising an antibody as claimed in any one of claims 1 to 12, together with a physiologically acceptable diluent or carrier.

15. The use of an antibody as claimed in any one of claims 1 to 12, in the manufacture of a medicament for use in a therapy which requires induction of tolerance to a therapeutic antibody.

Figure 1

# Schematic representation of a monovalent sFv-Ig minimal mutant




Note: Although this figure shows expression of the sFv in the VL-VH orientation, it is also possible to switch the orientation to VH-VL. In some cases this has been shown to increase expression levels.

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